Biochemical and Biophysical Research Communications 480 (2016) 261–267

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Crystal structure of the ADP-ribosylating component of BEC, the binary enterotoxin of *Clostridium perfringens*

Kazuki Kawahara [a, 1], Shinya Yonogi [b, c, 1], Ryota Munetomo [a], Hiroya Oki [a], Takuya Yoshida [a], Yuko Kumeda [b], Shigeaki Matsuda [c], Toshio Kodama [c], Tadayasu Ohkubo [a], Tetsuya Iida [c], Shota Nakamura [c, *]

[a] Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan
[b] Osaka Prefectural Institute of Public Health, Osaka, Osaka, Japan
[c] Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan

ARTICLE INFO

Article history:
Received 5 October 2016
Accepted 14 October 2016
Available online 15 October 2016

Keywords:
C. perfringens
Binary enterotoxin
Protein structure
X-ray crystallography

ABSTRACT

Binary enterotoxin of *Clostridium perfringens* (BEC), consisting of the components BECa and BECb, was recently identified as a novel enterotoxin produced by *C. perfringens* that causes acute gastroenteritis in humans. Although the detailed mechanism of cell intoxication by BEC remains to be defined, BECa shows both NAD^+-glycohydrolase and actin ADP-ribosyltransferase activities in the presence of NAD^+. In this study, we determined the first crystal structure of BECa in its apo-state and in complex with NADH. The structure of BECa shows striking resemblance with other binary actin ADP-ribosylating toxins (ADPRTs), especially in terms of its overall protein fold and mechanisms of substrate recognition. We present a detailed picture of interactions between BECa and NADH, including bound water molecules located near the C1′-N glycosidic bond of NADH and the catalytically important ADP-ribosylating turn-turn (ARTT) loop. We observed that the conformational rearrangement of the ARTT loop, possibly triggered by a conformational change involving a conserved tyrosine residue coupled with substrate binding, plays a crucial role in catalysis by properly positioning a catalytic glutamate residue in the E-X-E motif of the ARTT loop in contact with the nucleophile. Our results for BECa provide insight into the common catalytic mechanism of the family of binary actin ADPRTs.

1. Introduction

Clostridium perfringens is a well-known causative agent of various animal and human diseases, such as histotoxic and enteric infections, resulting from an ability of the organism to produce at least 17 different toxins reported to date [1,2]. The types and combinations of expressed toxins discovered are still increasing and expanding the known pathogenic consequences of *C. perfringens* infection [3–5]. The four major classical toxins, alpha, beta, epsilon, and iota toxins, classify *C. perfringens* isolates into five toxin types (A to E) [6,7]. C. *perfringens* enterotoxin (CPE) predominantly produced by *C. perfringens* type A isolates in sporulation is not included in the classification scheme, but is known as a main cause of human gastrointestinal illnesses, including food-borne gastroenteritis, antibiotic-associated diarrhea, and sporadic diarrhea [1–3]. In outbreaks of food-borne gastroenteritis due to *C. perfringens,* it is therefore important to detect CPE in patient fecal specimens and examine CPE production of *C. perfringens* isolates.

Recently, two food-borne gastroenteritis outbreaks occurred in Japan, which were caused by CPE-negative *C. perfringens* type-A isolates [5]. A novel enterotoxin, designated as binary enterotoxin of *C. perfringens* (BEC), distinct from CPE, was identified as the causative agent [5]. High-throughput genome sequencing revealed that BEC is composed of two proteins, BECa (~47 kDa) and BECb (~80 kDa), and has meaningful sequence homology with members of the binary ADP-ribosylating toxin (ADPRT)-family proteins, such as iota toxin from *C. perfringens* type E, C2 toxin from *C. botulinum*, *Clostridium difficile* toxin (CDT) from *C. difficile*, *Clostridium spiroforme* toxin (CST) from *C. spiroforme,* and vegetative insecticidal

* Corresponding author. Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 8318; fax: +81 6 6879 8372.
E-mail address: nshota@gen-info.osaka-u.ac.jp (S. Nakamura).
[1] Contributed equally.

http://dx.doi.org/10.1016/j.bbrc.2016.10.042

protein (VIP) from *Bacillus cereus* [5,8–12]. More recently, from another outbreak in Japan [13], the *C. perfringens* iota-like enterotoxin (CPILE), whose DNA sequence perfectly matched to BEC, was also identified as a possible source of CPE-negative *C. perfringens* type-A isolates, signifying the importance of understanding this novel enterotoxin in more detail.

Each binary ADPRT is composed of two separate components (an enzymatic A component and a cell-binding B component) [8,9], and mono-ADP-ribosylates globular actin during the cell-intoxication process, when both components work together [8–12]. Similar to cases observed with other binary ADPRTs, recombinantly expressed BEC has cytotoxic activity towards Vero cells, but only when both BECa and BECb are simultaneously administered [5]. Cytotoxicity resulting in cell rounding followed by cell lysis is primarily attributed to the actin ADP-ribosylating activity of BEC, which is clearly demonstrated by *in vitro* assays showing BECa has an actin ADP-ribosyltransferase activity in the presence of NAD^+ [5,8,12,13]. A sequence comparison showed that BECa possesses an aromatic residue-R/H, an E-X-E motif in the ADP-ribosylating turn-turn (ARTT) loop, and an S-T-S motif (second serine is threonine in BECa), which are highly conserved in the actin ADPRT family [14–18]. Based on these results, we concluded that the newly identified enterotoxin, BEC, is a binary enterotoxin that belongs to the actin ADPRT family.

In the present study, we determined the first crystal structure of BECa in its apo-state and in complex with NADH. The crystal structure of BECa showed remarkable similarity with other binary actin ADPRT-family members and reveals details of the substrate-binding mechanism of BECa, including implications for the mechanism underlying the molecular recognition of actin. Furthermore, we observed intriguing conformational variations of the catalytically important ARTT loop and several water molecules surrounding the substrate-binding pocket, both of which provide further insight into the precise functions and catalytic mechanisms of BECa.

2. Materials and methods

2.1. Crystallization

Expression and purification of BECa were performed as described previously [5]. Initial crystallization conditions were screened by sitting-drop vapor diffusion at 293 K, using the Crystal Screen 1 & 2 Formulation (Hampton Research). Crystallization drops were prepared by mixing 0.8 μL of protein solution (7.5 or 15 mg mL^{-1}) and 0.8 μL of reservoir solution and were equilibrated against 35 μL reservoir solutions. After several rounds of optimization, hexagonal crystals were obtained after 2 days from a mixture of 3 μL protein solution (7.5 mg mL^{-1}) and 2 μL reservoir solution consisting of 100 mM Tris-HCl (pH 8.8), 400 mM $MgCl_2 \cdot 6H_2O$, and 24% (w/v) polyethylene glycol 4000 at 293 K. A BECa-NADH crystal was grown by the co-crystallization method. The protein stock solution was diluted to 7.5 mg mL^{-1} and pre-incubated with the ligand NADH (10 mM) on ice. Using this solution, BECa-NADH was co-crystallized using the same conditions used for the BECa crystals. After optimization, hexagonal-shaped crystal of BECa-NADH was obtained in reservoir solution consisting of 100 mM Tris-HCl (pH 8.8), 450 mM $MgCl_2 \cdot 6H_2O$, and 23% (w/v) polyethylene glycol 4000 at 293 K.

2.2. Data collection and structure determination

Diffraction data from the BECa crystal were collected on an R-axis IV^{++} imaging plate detector using Cu-*Ka* X-rays from a Rigaku MicroMax-007 X-ray generator. The crystals were cryoprotected using reservoir solution and flash-cooled in a liquid nitrogen gas stream at 100 K. Data for the BECa crystal were processed with Crystal Clear software, version 1.3.5 (Rigaku) [19]. Diffraction data for the BECa-NADH complex crystal were collected at SPring-8 on beamline BL26B1 using a SaturnA200 CCD detector at 100 K. The data from the BECa-NADH crystal were processed with the

Table 1
Data collection and refinement statistics for the BECa and BECa-NADH structures.

	BECa	BECa-NADH
Data collection		
Wavelength (Å)	1.5418	1.0000
Space group	*I*222	*I*222
Unit cell dimensions		
a, *b*, *c* (Å)	69.85, 94.00, 121.06	70.34, 102.81, 125.03
α, β, γ (°)	90.0	90.0
Resolution range (Å)[a]	37.12–1.89 (1.96–1.89)	50.00–1.82 (1.85–1.82)
No. of unique reflections	31,752	40,199
Completeness (%)	98.4 (88.1)	99.8 (100.0)
Redundancy	9.5 (6.1)	7.0 (7.3)
$I/\sigma\langle I\rangle$	13.0 (4.2)	53.3 (10.8)
Overall B-factor from Wilson plot ($Å^2$)	23.5	22.7
R_{merge}[b]	0.095 (0.426)	0.073 (0.377)
Refinement		
R_{work}/R_{free}[c]	0.207/0.257	0.179/0.218
RMSD from ideal values		
Bond length (Å)	0.009	0.007
Bond angles (°)	1.034	0.827
Overall B-factors ($Å^2$)	30.0	31.0
Ramachandran plot statistics (%)		
Most favored	97.3	97.8
Allowed	2.7	2.2
Disallowed	0.0	0.0

[a] The values shown in parentheses are for the highest resolution shell.
[b] $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the *i* th measurement of an equivalent reflection and $\langle I(hkl)\rangle$ is the mean intensity for multiply recorded reflections.
[c] $R_{work} = \sum||F_o|-|F_c|| / \sum|F_o|$, where F_o and F_c are the calculated protein structure factors from the atomic model. R_{free} is calculated based on 10% randomly selected reflections not included in the calculation of the R_{work}.